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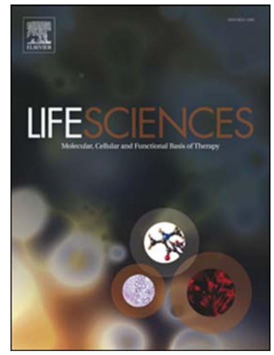

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The activation of melanogenesis by p-CREB and MITF signaling with extremely low-frequency electromagnetic fields on B16F10 melanoma

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structured abstract

Aims

Promote of melanogenesis by using specific frequencies of ELF-EMFs with B16F10 melanoma cells

Main methods

We examined the effect of ELF-EMFs according to various frequencies on B16F10 melanoma cells to investigate melanogenesis. B16F10 melanoma cells were stimulated at frequencies of 30 Hz, 50 Hz, 60 Hz, 75 Hz, and 100 Hz at an equal intensity of 2 mT for 3 days.

Key findings

The results show that 60–75 Hz ELF-EMFs significantly increase secreted melanin, cellular melanin content, and tyrosinase activity, and the cell mitochondria activity, cell viability, and cell membrane condition are unchanged. Furthermore, the protein expression level of MITF and p-CREB signaling pathway are significantly increased. Moreover, 60 Hz ELF-EMFs reduce the phosphorylate of ERK in B16F10 melanoma cells.

Significance

Optimal frequency of ELF-EMF is a new tool for safe hyperpigmentation therapy for an anti-gray hair treatment when melanin synthesis was reduced in the hair or hypopigmentary-related skin disorders such as vitiligo

Abstract

Melanin in the skin determines the skin color, and decreased melanin causes many hypopigmentation disorders and increased damage to skin by ultraviolet B (UVB) light irradiation. Here, we stimulate melanogenesis in B16F10 melanoma cells by using specific frequencies of ELF-EMFs. In this study, we focus on the melanogenesis of EMF-ELFs and find that 60–75 Hz ELF-EMFs upregulate melanin synthesis by stimulated expression of tyrosinase and TRP-1 through inhibition of phosphorylation ERK, activation of CREB, and MITF up-regulation in B16F10 melanoma cells. The results show that 60–75 Hz ELF-EMFs significantly increase secreted melanin, cellular melanin content, and tyrosinase activity, and the cell mitochondria activity, cell viability, and cell membrane condition are unchanged. Furthermore, the protein expression level of MITF and p-CREB signaling pathway are significantly increased. Moreover, 60 Hz ELF-EMFs reduce the phosphorylate of ERK in B16F10 melanoma cells. These findings indicate that stimulation of melanogenesis by using ELF-EMFs has therapeutic potential for treating hypopigmentation disorders such as vitiligo.

Key word: extremely low-frequency electromagnetic fields (ELF-EMFs), melanogenesis, p-CREB, MITF, B16F10 melanoma

Introduction

Melanogenesis is a physiological process resulting in the production of melanin pigment, which plays an important role in the prevention of sun-induced skin injury and contributes to skin and hair color[1]. UV-induced tanning can cause damage to DNA and other cellular molecules, leading to mutagenesis, carcinogenesis, altered immunological responses, and photoaging[2]. Melanin is a naturally synthesized polymer that protects the skin against the deleterious effects of ultraviolet (UV) radiation[3]. Currently, many natural compounds have been researched to induce melanogenesis, such as ardisia crenata extract[4], scoparone[2], mangosteen leaf extract[5], and kaliziri extract[6]. However, research on alleviating hyperpigmentation by noninvasive physical stimulation is scarce compared to natural compounds. Therefore, finding an innocuous method that can control melanogenesis would be invaluable in the cosmetic and medical fields. Photochemotherapy with psoralen plus ultraviolet A was the most popular treatment for vitiligo across the world until a few years ago[7]. To increase melanin synthesis, many physical treatment methods were attempted. Low-energy helium-neon laser (632.8 nm) irradiation clearly stimulates melanocyte proliferation and mitogen release for melanocyte growth and rescues damaged melanocyte, thereby providing a microenvironment for repigmentation in vitiligo[8]. Gu et al. reported that narrowband UVB increased melanogenesis-related gene expression [9], and broadband ultraviolet B (wavelengths from 290 to 320 nm) was widely used in the past for the treatment of various skin disorders include vitiligo[7]. Depending on the cell types, various stimulation techniques have been applied to activate cells. Among the methods of stimulation (cyclic pressure, cyclic compressive load, uniaxial strain, perfusion, shear and compression, ultrasound, laser, electrical stimulation, electromagnetic field, etc.) that affect cell activation, physical stimulation has been investigated extensively. As well, to increase cellular activity, many stimulation devices have been designed and are used clinically. Recently, electromagnetic fields (EMF) have been a major focus of scientific interest because of their potential influence on living organisms, and EMFs have emerged as a good tool for cell differentiation and cell therapy because of their invasive and nontoxic properties [10]. Especially, extremely low-frequency electromagnetic fields (ELF-EMF) influence cell proliferation[11, 12] and enhanced osteogenic differentiation [13]. Furthermore, one investigator reported that ELF-EMFs induce neural differentiation of human bone marrow-derived mesenchymal stem cells[10, 14]. Such studies show that ELF-EMFs affect cell function through mechanical actions on both intracellular and membrane proteins, which includes ion channels, membrane receptors, and enzymes[15].

Our research applies ELF-EMFs to increase melanogenic activity. The biosynthesis of melanin is a complicated process involving many factors. The melanogenic process is modulated by enzymatic cascades including tyrosinase, tyrosinase-related protein (TRP)-1, and their transcription factors such as microphthalmia-associated transcription factor (MITF), cAMP response element binding protein (CREB), and extracellular-regulated kinase (ERK)[16]. Alpha-melanocyte stimulating hormone (α-MSH), which induces MITF, is the most important hormone in stimulating melanogenesis. α-MSH binds to melanocortin 1 receptors, which causes cAMP production, and cAMP leads to phosphorylation of the CREB transcription factor, which in turn promotes MITF activation. MITFs bind to the promoter regions of melanin production genes and positively regulate their transcription of TRP-1 and tyrosinase[17-19]. Also, the ERK pathway appears to influence the synthesis of melanin via a negative feedback mechanism involving cAMP[20].

In this study, we examined the effect of ELF-EMFs according to various frequencies on B16F10 melanoma cells to investigate melanogenesis. B16F10 melanoma cells were stimulated at frequencies of 30 Hz, 50 Hz, 60 Hz, 75 Hz, and 100 Hz at an equal intensity of 2 mT for 3 days. To confirm the melanogenesis, we performed melanin secretion assay, melanin contents assay, intracellular tyrosinase assay, western blot analysis, and immunohistochemical staining. In particular, we analyzed changes in the ERK and CREB signaling associated with MITF regulation by ELF-EMFs.

Materials and Methods

Cell cultured

B16F10 melanoma cells (ATCC CRL-6475;BCRC60031) were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Welgene, Korea), 50 units/mL penicillin, and 50 µg/mL streptomycin (Hyclone, USA). The cells were then incubated in an atmosphere of 5% CO_2 at 37 °C. Cells were culture in a 35 mm-diameter tissue culture plate, and ELF-EMF treatment begin 24 h after the cells were seeded.

ELF-EMF exposure

We used a Helmholtz coil, which is able to generate magnetic fields; the apparatus is depicted in Fig. 1. The stimulus intensity was 2 mT, and stimulus wave was in pulse form. The electromagnetic field device was placed in a 37 °C incubator at 5% humidified atmosphere, and B16F10 melanoma cells were stimulated by ELF-EMFs at frequencies of 30 Hz, 50 Hz, 60 Hz, 75 Hz, and 100 Hz for 3 days. Cells that were not stimulated with ELF-EMFs served as the negative and positive controls, which were treated with α-MSH.

Mitochondria activity assay

Cell mitochondria activity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. B16F10 melanoma cells were seeded in 12-well dishes at a density of 5×10^4 cells per well. After 24 h, different frequencies of ELF-EMFs were stimulated, and the cells were incubated for 72 h. Then, 100 µl of MTT solution (5 mg/ml in PBS, Sigma) were added into each well, and the cells were incubated in a 37 °C incubator for 4 h followed by the addition of dimethyl sulfoxide (DMSO, Sigma) to dissolve the formazan crystals, and the plates were gently shaken for 5 min. The optical absorbance of each well was measured at 570 nm with a spectrophotometer (Spectrum Analyzer. Victor 1420-050, PerkinElmer Life Science, Turku, Finland).

Cell proliferation

To test the effect of ELF-EMFs on the proliferation of B16F10 melanoma, cell counting was used. B16F10 melanoma cells were counted using a scepter automated cell counter (Millipore, Billerica, MA, USA) based on the instructions of the manufacturer.

Cell Cytotoxicity-Lactate Dehydrogenase (LDH) assay

Cell membrane integrity was assessed by estimating the amount of LDH present in the cell culture media. The cytosolic enzyme LDH will be released owing to management of the cell membrane[21]. We used an LDH-LQ kit (Asan Pharmaceutical Inc., Korea) for measuring LDH activity. After 3 days of culture, 100 µl of cell culture media was transferred to Ep tubes and centrifuged at 2000 rpm for 5 min. Fifty microliters of working solution was added to all Ep tubes and incubated in the dark at room temperature for 30 min. The reaction was completed by adding 1 N hydrogen chloride, and the absorbance was measured spectrophotometrically at 570 nm.

Measurement of melanin secretion

A secreted melanin assay was performed using a previously described method[5]. B16F10 melanoma cells were seeded in a 35 mm-diameter tissue culture plate at a density of 1×10^5 cells per well and stimulated with or without ELF-EMFs for 3 days. After 3 days, the culture medium was harvested and centrifuged at 10,000 rpm for 10 min. Absorbance was measured at 405 nm using a spectrophotometer

Measurement of melanin content

The amount of intracellular melanin content synthesized by cultured B16F10 melanoma cells treated with or without ELF-EMFs was quantified as previously described[5] with slight modification. The B16F10 melanoma cells were seeded in a 35 mm-diameter culture dish at a density of 1×10^5cells and incubated overnight to allow the cells to adhere. After 24 h, the cells were treated with or without ELF-EMFs. After 3 days, the growth medium was eliminated, and the cells were washed with phosphate-buffer saline (PBS) and then solubilized with 10% DMSO, dissolved in 1M NaOH (95 °C), and boiled for 2 h to solubilized the melanin. The intracellular melanin concentrations were measured with a spectrophotometer at 400 nm and compared to an absorbance value of negative control, which is untreated with α-MSH and ELF-EMFs.

Intracellular tyrosinase assay

Tyrosinase activity was estimated from the rate of production of dopachrome from L-DOPA as previously reported[6] with slight modifications. The B16F10 melanoma cells were seeded in a 35 mm-diameter culture dish at a density of 1×10^5 cells and allowed to attach for 24 h; then, the cells were treated with or without ELF-EMFs for 3 days. The cells were washed with PBS twice and harvested by trypsinization (0.25% trypsine/0.02% EDTA in PBS; Sigma). The cells were collected in an Ep tube and centrifuged at 3,000 rpm for 5 min. The pelleted cells were washed once again with PBS, and then 200 µl of Tris-0.1% Triton X-100 (pH 6.8) were added to each Ep tube. All tubes were incubated at -20 °C for 1 h, and then the lysates were centrifuged at 13,000 rpm for 10 min to obtain the supernatant for the intracellular tyrosinase activity assay. Protein concentrations were measured by bicinchonic acid (BCA; Thermo Fisher Scientific, USA) protein assay with bovine serum albumin (BSA) as a standard. One hundred eighty microliters of supernatant containing 50 µg of protein was placed in each well of a 96 well plate and then added with L-DOPA (20 µl; Sigma) in sodium phosphate buffer (10 mM). After incubation at 37 °C for 1 h, the generated dopachrome was measured at the absorbance of 475 nm. The amount of dopachrome was expressed to prevent change relative to the negative control.

Western blotting

B16F10 melanoma cells were seeded in a 35 mm-diameter culture dish at a density of 1×10^5 cells and incubated for 3 days as described above. The medium was removed, and the cells were washed twice with PBS and then lysted in PBS containing 10% glycerol, 5% β-mercaptoechanol, 2% sodium dodecyl sulfate (SDS), and 0.01% bromophenol blue in a 62.6 mM Tris-HCl buffer (pH 6.8); the cell lysates were then denatured at 100 °C for 5 min. The total protein content of cell lysates was determined using the BCA protein assay, and protein amounts in each sample (40 µg total protein) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then the fractionated protein in SDS-PAGE was electrotransferred from the gel onto the nitrocellulose membrane (Millipore Co., Massachusetts). The membranes were blocked with 5% fat-free skim milk in tris-buffered saline (TBS) containing 0.1% Tween20 (TBS-T buffer) at room temperature for 1 h. After washing three times with TBS-T, the membrane was incubated overnight with the primary antibodies diluted in 10% bovine serum albumin: anti-rabbit β-actin antibody (1:1000), anti-goat MITF antibody (1:500), anti-rabbit extracellular signal-regulated kinase (ERK) antibody (1:1000), anti-rabbit p-ERK antibody (1:1000), anti-rabbit cyclic AMP response element binding protein (CREB) antibody (1:1000), anti-rabbit p-CREB antibody (1:1000), anti-goat tyrosinase antibody (1:1000), and anti-rabbit tyrosinase-related proteins (TRP)-1 antibody (1:1000). The primary antibodies were removed, the membranes were washed three times with TBS-T buffer, and incubation with horseradish peroxidase (HRP) conjugated anti-rabbit (cell signaling) or anti-goat (Santa Cruz) secondary andibodies for 3 h at room temperature. The membrane was washed extensively in TBS-T to remove any excess secondary antibodies, the blot was visualized with enhanced chemiluminescence reagent (Thermo Fisher Scienctific, USA) and photographed using a gel imaging system, ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA). The western blot assays are representative of at least three experiments, and the results were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Fontana-Masson silver staining

To assess melanin content in B16F10 melanoma cells, we performed densitometric analysis of Fontana-Masson silver staining. The Fontana-Masson silver staining was performed using a previously described method[22] with formalin fixed slides stained with silver nitrate (Kojima Chemical, Kashiwabara, Japan) for 1 h at 56 °C and washed with distillated water. Then, the slides were fixed in 5% sodium thiosulfate solution (Duksan, Seoul, Korea) for 5 min and washed with distilled water. Next, they were stained with nuclear faster red solution (Flka, Buchs, Switzerland) for 5 min and washed with distillated water three times. Finally, after dehydration with 95% ethanol and 100% ethanol, the slides were washed with xylene (Duksan) two times.

Statistical Analysis

Data was analyzed by one-way analysis of variance (ANOVA) and student t-test. When the value of p was <0.05, the difference between means was considered significant (*p<0.05,). Graphical representations were produced with the help of Sigmaplot 2001 software.

Result

Morphology of B16F10 melanoma cells

B16F10 melanoma cells treated at various frequencies of ELF-EMFs were incubated for 3 days and compared to the ELF-EMF untreated cells shown in figure 2. The group exposed to ELF-EMFs showed that cells were arranged in a linear array and a greatly dendritic network between cells compared to the control and α-MSH groups. In addition, compared to the control group cells and the ELF-EMF treated group, the necrosis and morphological features of the apoptosis of cells was not observed after exposure to ELF-EMFs. Therefore, ELF-EMF exposure does not induce cytotoxicity.

Cell number counting and mitochondrial activity

To test the effect of ELF-EMFs on the cell viability of B16F10 melanoma cells, cell counting was performed using a Scepter automated cell counter after cell culture. After ELF-EMF exposure, the total cell numbers of all groups increased more than the seeding cell numbers. In figure 3A, cell numbers were as follows 3 days after treatment with ELF-EMFs: 2.72×10^5 cells in the negative control group, 3.02×10^5 cells in the α-MSH treated group that added α-MSH, 2.70×10^5 cells in the group stimulated with 30 Hz, 2.71×10^5 cells in the group treated with 50 Hz, 2.74×10^5 cells in the group receiving 60 Hz, 2.80×10^5 cells in the group exposed to 75 Hz, and 2.75×10^5 cells in the group treated with 100 Hz. As a result of cell counting, ELF-EMFs did not have a cytotoxic effect on B16F10 melanoma cells and did not cause cell apoptosis or necrosis compared to the control group. The cellular mitochondrial activity of B16F10 melanoma cells was measured by an MTT assay (figure 3B). The result of the MTT assay shows that cell mitochondrial activity of six experimental groups were similar, and ELF-EMFs did not affect the cell mitochondrial activity. Our data showed that 3 days of stimulus did not decrease the mitochondrial activity of B16F10 melanoma cells.

Cytotoxicity-Lactate Dehydrogenase (LDH) assay

LDH is a cytoplasmic enzyme released when the cell membranes are damaged that is assessed in cell culture medium supernatants, and the LDH leakage assay is a simple and fast cytotoxicity assay based on the measurement of lactate dehydrogenase activity in an extracellular medium[21]. The membrane damage to B16F10 melanoma cells after treatment with ELF-EMF was measured by the release of LDH. The control cells and cells treated with ELF-EMFs showed a similar amount of LDH secretion. As a result of LDH assay in figure 4, ELF-EMFs did not influence the damage of cell membranes.

Melanin secretion assay

To measure whether the ELF-EMFs lead to melanogenesis, the amount of secretion of melanin into the cell culture was measured. The α-MSH is known as cAMP evaluating agent, because the cAMP pathway is one of the most pivotal signaling pathways in melanogenesis[5]. Thus, α-MSH is effective in melanogenesis, so α-MSH is used as a positive control in this experiment. Figure 5 shows that melanin secretion levels significantly increased in cells treated with α-MSH (1.4-fold) and 60 Hz and 75 Hz treatment by ELF-EMFs in B16F10 melanoma cells (1.5-fold) compared to the negative control. The result showed that 60 Hz and 75 Hz were effective for melanogenesis in B16F10 melanoma cells.

Melanin content assay

The amount of intracellular melanin in B16F10 melanoma cells treated with or without ELF-EMFs was quantified. Figure 6A shows that melanin content increased in all groups exposed to ELF-EMFs cells. The 30 Hz group rose 1.65-fold, the 50 Hz group was enhanced ~1.7-fold, and the 75 Hz and 100 Hz groups increased approximately 1.8-fold over the negative control group. In particular, 60 Hz ELF-EMFs cause a 2.4-fold increase in melanin content of cells compared to the negative control and ~1.3-fold more than the α-MSH group. These results suggest that ELF-EMFs, especially at 60 Hz, also up-regulated intracellular melanin synthesis.

Tyrosinase activity assay

Tyrosinase is the most important enzyme in melanin biosynthesis. Therefore, the effects of ELF-EMFs on intracellular tyrosinase activity were assessed in B16F10 melanoma cells (figure 7). Compared to treatment with medium only (negative control group), treatment with ELF-EMFs of various frequencies increased tyrosinase activity in B16F10 melanoma cells. Among these ELF-EMF groups, the tyrosinase activity of 50 Hz groups was enhanced 1.27-fold, and the 100 Hz group rose to 1.36-fold more than the negative control group. In particular, treatment at 60 Hz showed strongly increased tyrosinase activity compared to the negative group (1.8-fold) and slightly stronger tyrosinase activity than the positive control group (1.2-fold). It was observed that ELF-EMFs promoted melanogenesis in B16F10 melanoma cells.

Melanogenic enzyme expression in B16F10 melanoma cells

Melanin biosynthesis is catalyzed by two major enzymes: tyrosinase and TRP1. The expression of these enzymes was determined by western blotting using specific antibodies. The tyrosinase expression level increased approximately 1.3-fold in the 30 Hz and 50 Hz groups. Especially, the 60 Hz (1.9-fold) and 75 Hz (2.3-fold) groups among the groups exposed to ELF-EMFs had very strong expression compared to the control group. As shown in figure 8, TRP-1 expression levels increased in all frequency groups of ELF-EMFs. TRP-1 expression levels increased 5.3-fold in the 30 Hz group, 8.9-fold in the 50 Hz group, and 7.2-fold in the 100 Hz group. In particular, the 60 Hz (13.7-fold) and 75 Hz (14.5-fold) groups had very strong expression compared to the control group and an approximately 1.3-fold increase compared to the α-MSH group. These results suggested that melanogenesis-related protein expression, tyrosinase, and TRP-1 were strongly up-regulated by exposure to 60 Hz and 75 Hz ELF-EMFs.

To clarify the signaling of ELF-EMFs in the synthesis of melanin, we examined the phosphorylation of ERK and CREB and the activation of MITF, which is related to tyrosinase and TRP-1 expression. The expression levels of MITF and total and phosphorylated ERK and CREB were measured by western blotting and the J-imaging program. As shown in figure 8, the p-CREB activation level was increased 1.6-fold and the MITF expression level was increased 1.3-fold over the control group. p-ERK activation was decreased at 50 Hz and 60 Hz among the ELF-EMF exposure groups. In particular, there are specific suppressions in the 60 Hz groups (0.4-fold). The result of western blotting showed that melanogenesis-related enzyme, tyrosinase, and TRP-1 was up-regulated in B16F10 melanoma stimulated by 60 Hz ELF-EMFs. Furthermore, 60 Hz ELF-EMFs induced the upstream of MITF and p-CREB levels and down-regulate p-ERK signaling molecules (figure 8).

Thus, the result implies that treatment of 60 Hz ELF-EMFs induced melanogenesis via MITF and phosphorylation ERK and CREB.

Fontana-Masson sliver staining

To visualize the melanin, the cells were stained with fontana-masson stain. Figure 9 shows the melanin content determined by Fontana-Masson staining. Silver nitrate (AgNO3) reacts with melanin to produce metallic silver (Ag), resulting in a black stain that can be visualized with a light microscop[23]. As shown in figure 9, compared to the controls, the amount of melanin granules were significantly increased and stained by ELF-EMF(dark brown color, arrow). The 60 Hz and 75 Hz ELF-EMF groups had markedly strongly induced formation of the pigment. The result showed that the staining intensity per cells was analogize with the result of above measurement experimental result such as western blotting, Tyrosinase activity assay, melanin content and melain secretion assay. Relative staining intensity was scored on a light microscopy image by means of the following, arbitrary, intensities: no or weak staining (-), low intensity (+), moderate intensity (++), and strong intensity (+++). The relative staining intensity was assessed by light microscopy (Table 1).

Discussion

Recently, ELF-EMF was especially studied by many researchers. Although magnetic energies are low, cell studies showed that low-frequency EMFs interact with biological systems and may have health effects[24], and ELF-EMFs have a significant function in cell cycle regulation, proliferation, differentiation, mitoses, apoptosis or stress regulation, and induced genes[25]. Some investigators discovered that ELF-EMFs affect cell function through mechanical action on both intracellular and membrane proteins, which includes ion channel, membrane receptor, and enzyme[15]. In spite of the mechanism of EMFs still under research, all above studies agree on the effect of ELF-EMFs.

In our research, we apply ELF-EMFs to the stimulation of melanogenesis in B16F10 melanoma cells. We first examine the cytotoxicity of ELF-EMFs. To determine the cytotoxicity of ELF-EMFs on B16F10 melanoma cells, the cells were treated with ELF-EMFs at various frequencies for 3 days, and the cells were analyzed using MTT assay and cell number counting. Many reports already demonstrated the nontoxicity of ELF-EMFs [26, 27], and our research result further indicated that ELF-EMFs do not have a cytotoxic effect on B16F10 melanoma cells in any frequency condition (figure 3.4). In addition, ELF-EMFs do not sustain damage to B16F10 melanoma cell membrane, which is verified by lactate dehydrogenase release assay (figure 5).

So at this studies, histological change due to pigmenting effect of ELF-EMFs was observed using Fontana-Masson staining (figure9). As shown in our results, 60 Hz and 75 Hz ELF-EMFs clearly increased the formation of pigment, but did not melanin formation in the control, 30 Hz and 100 Hz ELF-EMF groups, which was well observed by Fontana-Masson staining.

Melanin biosynthesis is catalyzed by two major enzymes: tyrosinase and TRP1. The expression of these enzymes was determined by western blotting using specific antibodies, and the result showed that treatment with 60 Hz and 75 Hz of EMF induced the expression of tyrosinase and TRP-1 (figure 8). As shown in figure 8A and 8B, melanogenesis-related protein expression, tyrosinase, and TRP-1 were strongly up-regulated by exposure to 60 Hz and 75 Hz of ELF-EMFs.

Previous studies demonstrated that a skin whitening agent can activate ERK phosphorylation and reduce MITF and p-CREB protein expression to decrease tyrosinase synthesis in α-MSH-induced melanogenesis in B16F10 melanoma cells[28, 29]. Generally, the expression of melanogenic-related enzyme genes are regulated by MITF, which binds on the regulatory element of tyrosinase and TRP-1 genes[30], and related studies have demonstrated that the phosphorylatioates activate the transcription factor CREB, resulting in an induction of MITF expression via binds and activating the MITF promoter according to the cyclic adenosine monophosphate (cAMP) response element[28, 29, 31].

Thus, to clarify the signaling pathway of ELF-EMFs in the synthesis of melanin, we examined the phosphorylation of ERK and CREB and the activation of MITF, which is related to tyrosinase and TRP-1 expression. The results showed a significant decrease in the activation of ERK at 60–75 Hz of ELF-EMFs, which can also lead to stimulation of the melanogenic pathway by accelerating MITF activation. In addition, the results showed that 60 Hz of ELF-EMF treatment significantly induced phosphorylation of CREB, which also led to the activation of MITF expression (figure 8B). This MITF is an important transcription factor in the regulation of tyrosinase activity and expression of TRP-1[32], and MITF binds to the promoter regions of melanin product genes and positively regulates their transcription such as tyrosinase and TRP-1[17-19]. Previous studies showed that a whitening agent can activate ERK phosphorylation and reduce MITF and p-CREB protein expression to decrease tyrosinase synthesis in α-MSH-induced melanogenesis in B16F10 melanoma cells, so the expression of the melanogenic enzyme's genes are regulated by MITF, which binds onto the regulatory element of tyrosinase and TRP-1 genes[28-30].

ERK regulates cell growth, differentiation, and survival and is also an important regulator of the melanogenic process [33], and then it was well kwnon that the inhibition of ERK expression increases melanin synthesis[34, 35]. Also, enerelt et al reported that p-CREB protein expression was significantly increased after EMF exposure on human bone marrow mesenchymal stem cells[36] and CREB phosphorylation was increased in response to ELF-EMF in vitro[14, 37]. The phosphorylated active form of CREB binds and activates MITF, which in turn stimulates the transcription of the key melanogenic enzyme, such as TRP-1 and tyrosinase[38]. Form those studies above, the results imply that treatment with 60 Hz ELF-EMFs influenced mechanically sensitive kisnase such as ERK and CREB[14, 39]. Our research points to melanogenesis regulation of the expression of CREB, ERK, and MITF because CREB, ERK, and MITF play a critical role in regulating the melanogenic pathway[40].

The Fontana-Masson stain is a histochemical technique that oxidizes melanin and melanin-like pigments as it reduces silver, and it is commonly employed to measure pigmentation effects such as skin whitening, tanning, and hyperpigmentation disorder[23]. Lee et al reported that the whitening agent was associated with a reduction in the levels of MITF and TRP-2 expression, and it had a greater effect histopathologically in melanin reduction shown by a Fontana-Masson stain[41]. On the basis of this result, at this studies show that ELF-EMFs at the specific frequency can stimulate pigmentation of B16F10 melanoma cells.

Conclusion

Currently, there are insufficient commercial products for induction of hyperpigmentation[40]. In this study, we investigated whether the frequency of ELF-EMFs has an effect on hypopigmentation. Our data suggest that 60–75 Hz ELF-EMFs stimulate the biosynthesis of melanin by promoting tyrosinase and TRP-1, which are mediated through activation of CREB, MITF, and a reduction of phosphorylation ERK. These results may indicate that the optimal frequency of ELF-EMF is a new tool for safe hyperpigmentation therapy for an anti-gray hair treatment when melanin synthesis was reduced in the hair or hypopigmentary-related skin disorders such as vitiligo. Future studies will focus on ELF-EMF-induced melanogenesis in a three-dimensional culture model and in vivo.

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Figure 1

Image of the EMF stimulation model in CO_2 incubator. We used continuous pulsed EMFs (2 mT) for experiments.

Figure 2

Morphological changes of B16F10 melanoma cells during EMF treatment. The cells were cultured for 3 days with or without EMF stimulation. There is no significant visible differences in EMF treated cells. (bar = 100 μm) Cultured in normal medium (A), α-MSH (B), 30 Hz (C), 50 Hz (D), 60 Hz (E), 75 Hz (F), 100 Hz (G) . (X100).

Figure 3

B16F10 melanoma cells were seeded in a 35 mm tissue culture plate, and their proliferation was measured on the 2 days after EMF by counting cell numbers (A). The effect of mitochondrial activity on B16F10 melanoma cells by EMF treatment. Cells were cultured for 2 days with or without EMF stimulation. Mitochondrial activity was determined by the MTT reduction assay. Each percentage value in the EMF-treated cells was calculated with respect to that in the untreated cells (B). (n = 3)

Figure 4

Cell membrane damage to B16F10 melanoma cells after treatment with EMF was measured by the release of LDH. LDH release to the medium is a measurement of cell death due to cell membrane damage. The EMF treatment provoked a similar release of LDH activity with EMF untreated cells. (n = 3)

Figure 5

Effect of EMF on melanin secretion on media in B16F10 melanoma cells. The percentage values of the treated cells are expressed relatively compared to the control cells. α-MSH was used as a positive control for melanin release. (*$p<0.05$)

Figure 6

Effect of EMF on melanin synthesis in B16F10 melanoma cells (A). Lysates of cells treated with or without EMF (B). (*$p<0.05$)

Figure 7

Effect of EMF on intracellular tyrosinase activity. B16F10 melanoma cells were incubated without (control) and with EMFs for 2 days. Promotion of intracellular tyrosinase activity by EMF at different frequencies. Result were expressed as percentages relative to control and for three separate experiments. (*$p<0.05$)

Figure 8

Effect of EMF on the protein levels of tyrosinase, TRP-1, p-CREB, CREB, p-ERK, ERK, MITF, and β-actin in B16F10 melanoma cells. The cells were treated with or without EMF for the indicated times. Western blotting analysis was performed to examine melanogenesis-related protein expression levels (A). The graph indicates the expression level against the β-actin expression level (B).

Figure 9

Melanin was visualized by Fontana-Masson silver staining. Melanin is stained by dark black. (Original magnification X100. bar = 100 μm)

Fig. 1

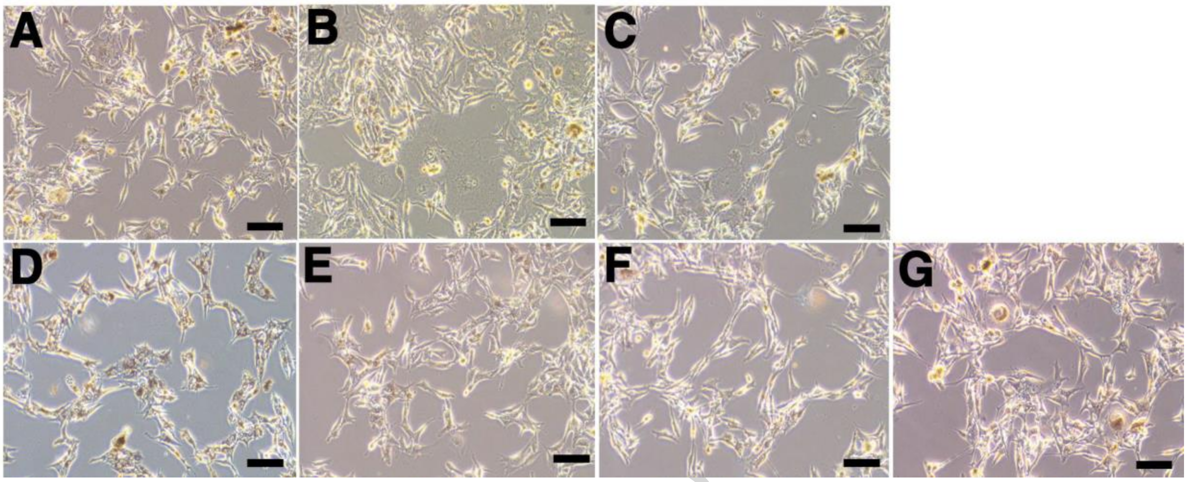


Fig. 2

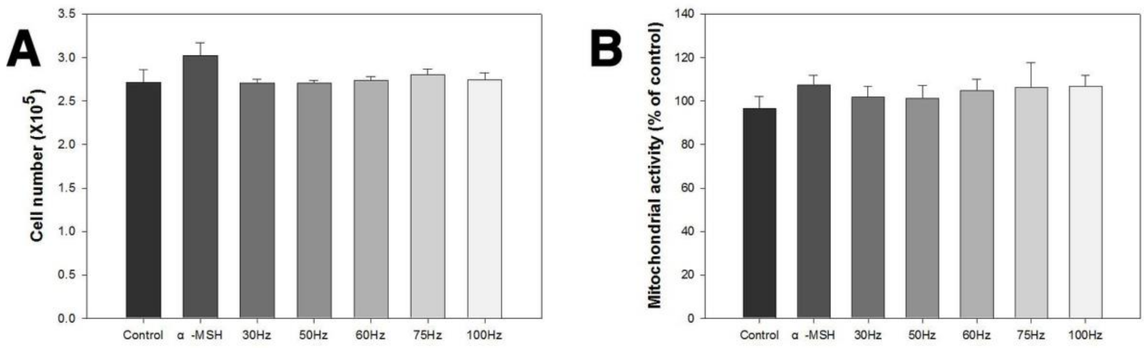
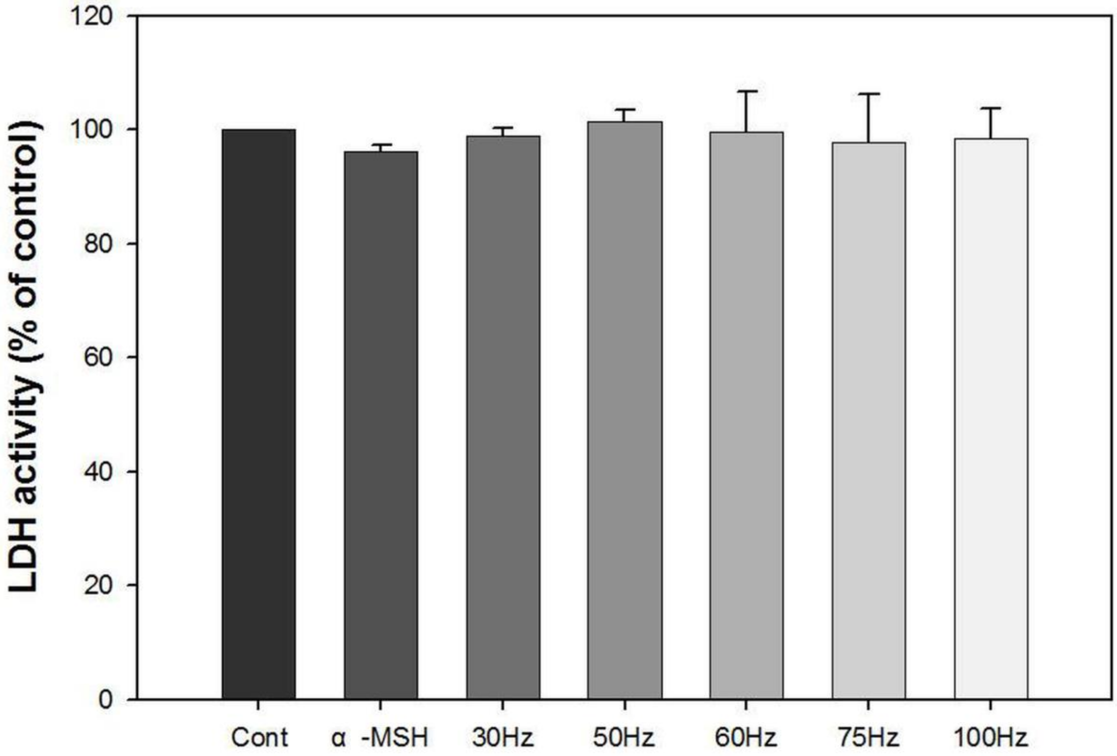
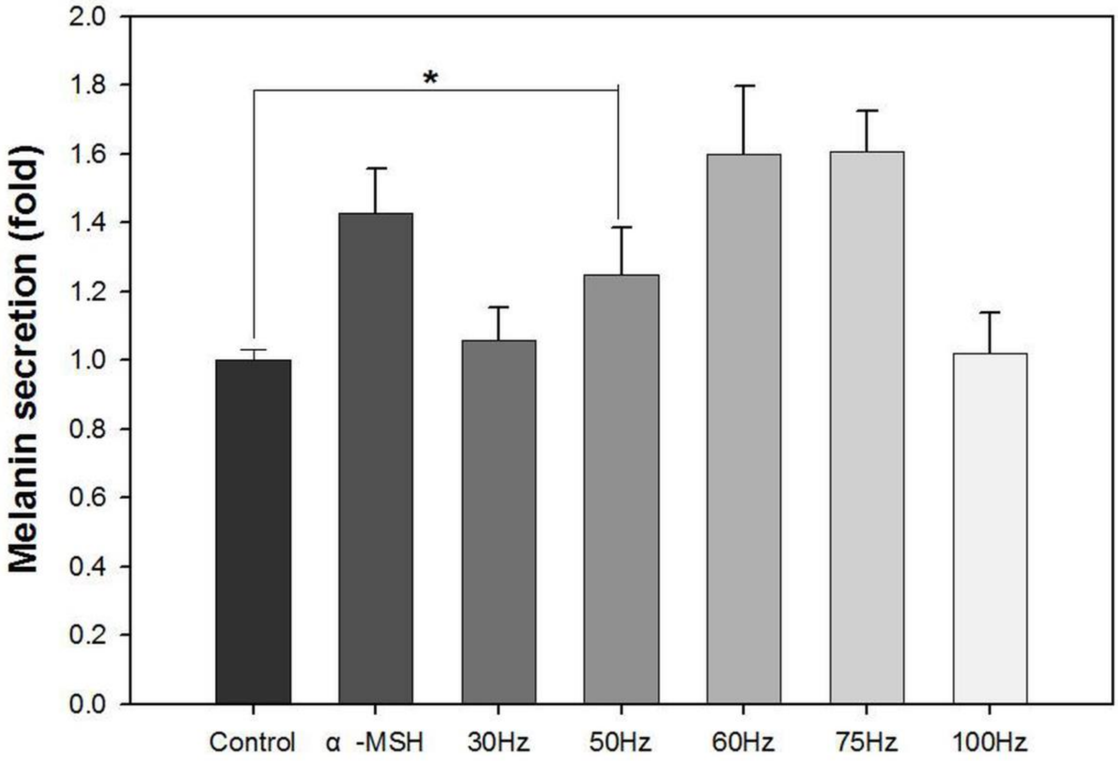

A
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Cell number (X10^5)
Control
α -MSH
30Hz
50Hz
60Hz
75Hz
100Hz
B
140
120
100
80
60
40
20
0
Mitochondrial activity (% of control)
Control
α -MSH
30Hz
50Hz
60Hz
75Hz
100Hz

Fig. 3



LDH activity (% of control)
120
100
80
60
40
20
0
Cont
α -MSH
30Hz
50Hz
60Hz
75Hz
100Hz

Fig. 4



Melanin secretion (fold)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
α -MSH
30Hz
50Hz
60Hz
75Hz
100Hz

Fig. 5

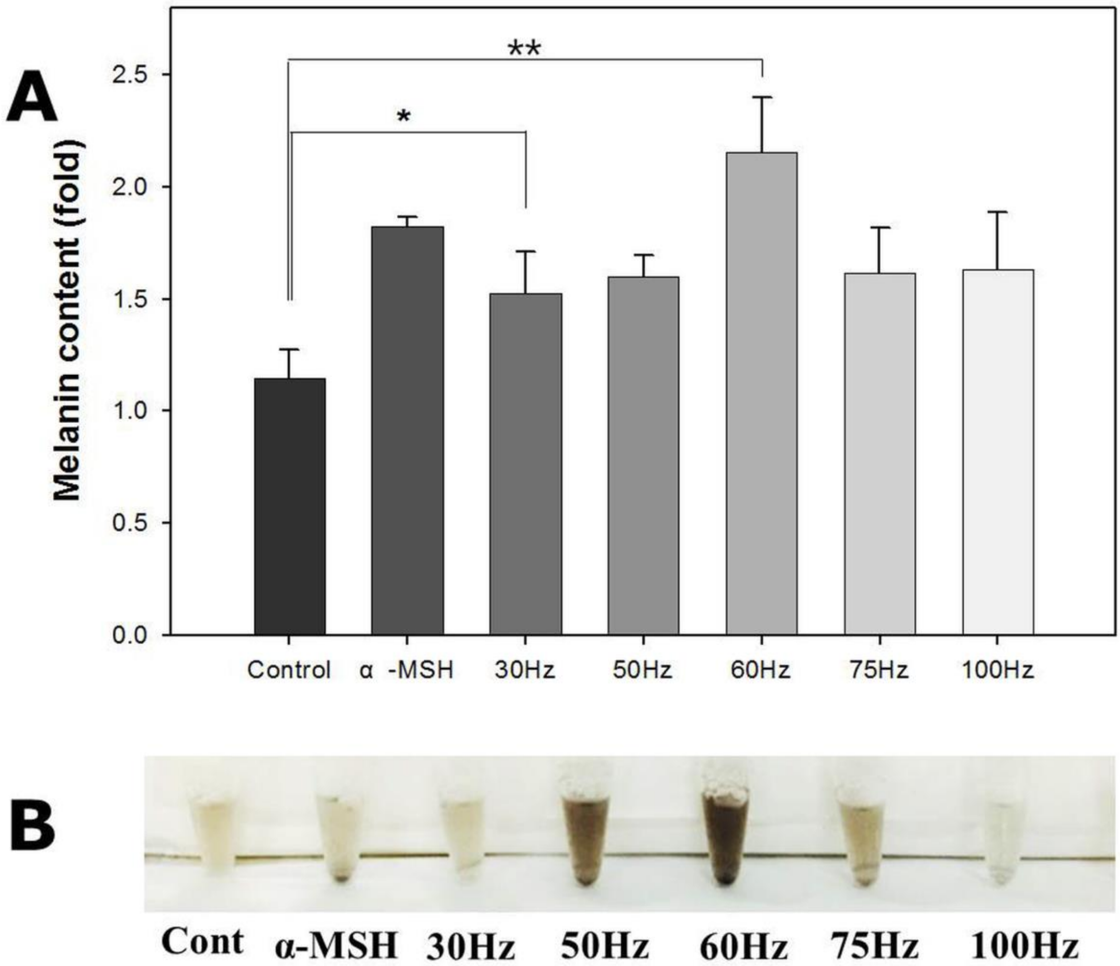
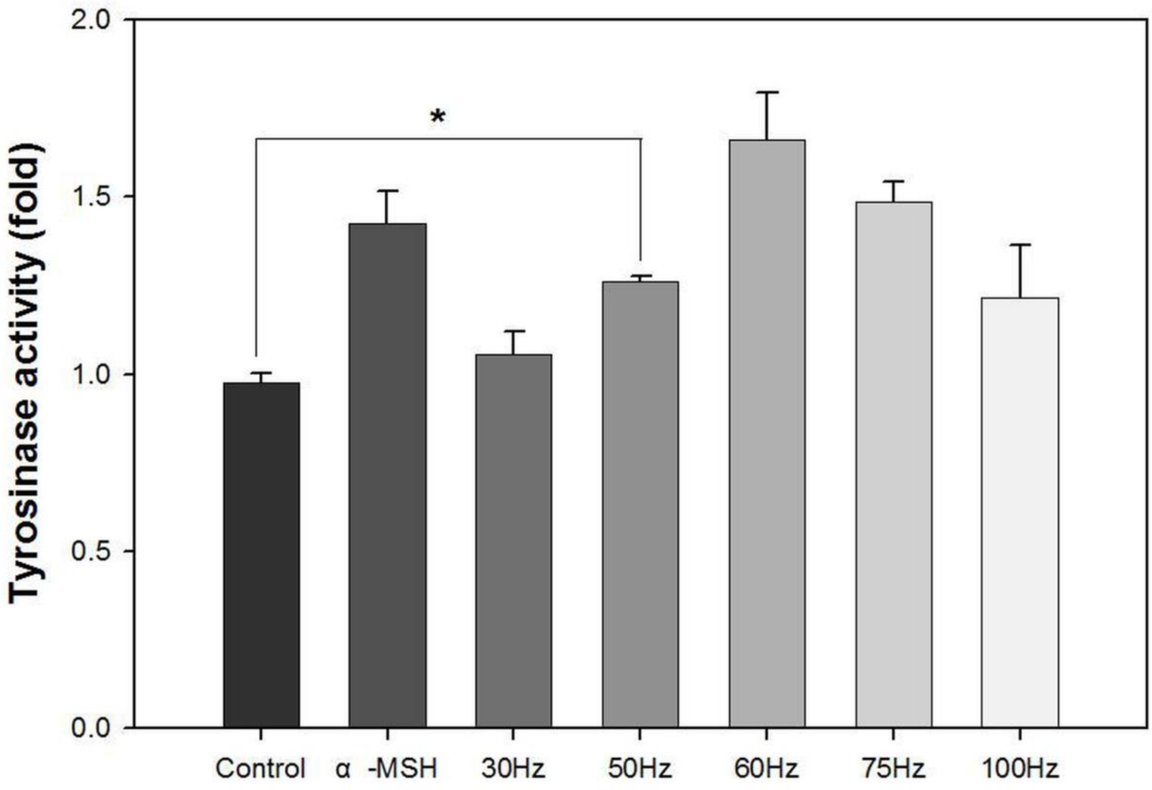

A
Melanin content (fold)
0.0
0.5
1.0
1.5
2.0
2.5
**
*
Control
α -MSH
30Hz
50Hz
60Hz
75Hz
100Hz
B
Cont
α-MSH
30Hz
50Hz
60Hz
75Hz
100Hz

Fig. 6



2.0
1.5
1.0
0.5
0.0
Tyrosinase activity (fold)
*
Control
α -MSH
30Hz
50Hz
60Hz
75Hz
100Hz

Fig. 7

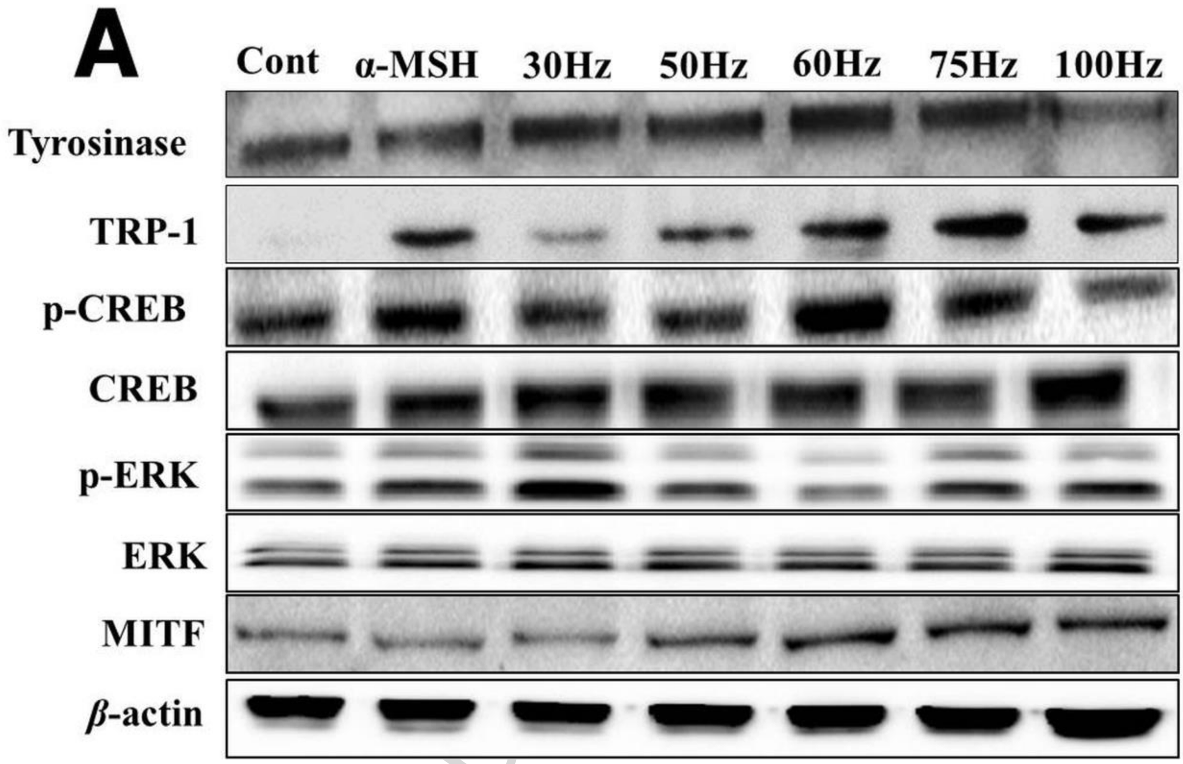

A
Cont
α-MSH
30Hz
50Hz
60Hz
75Hz
100Hz
Tyrosinase
TRP-1
p-CREB
CREB
p-ERK
ERK
MITF
β-actin

Fig. 8A

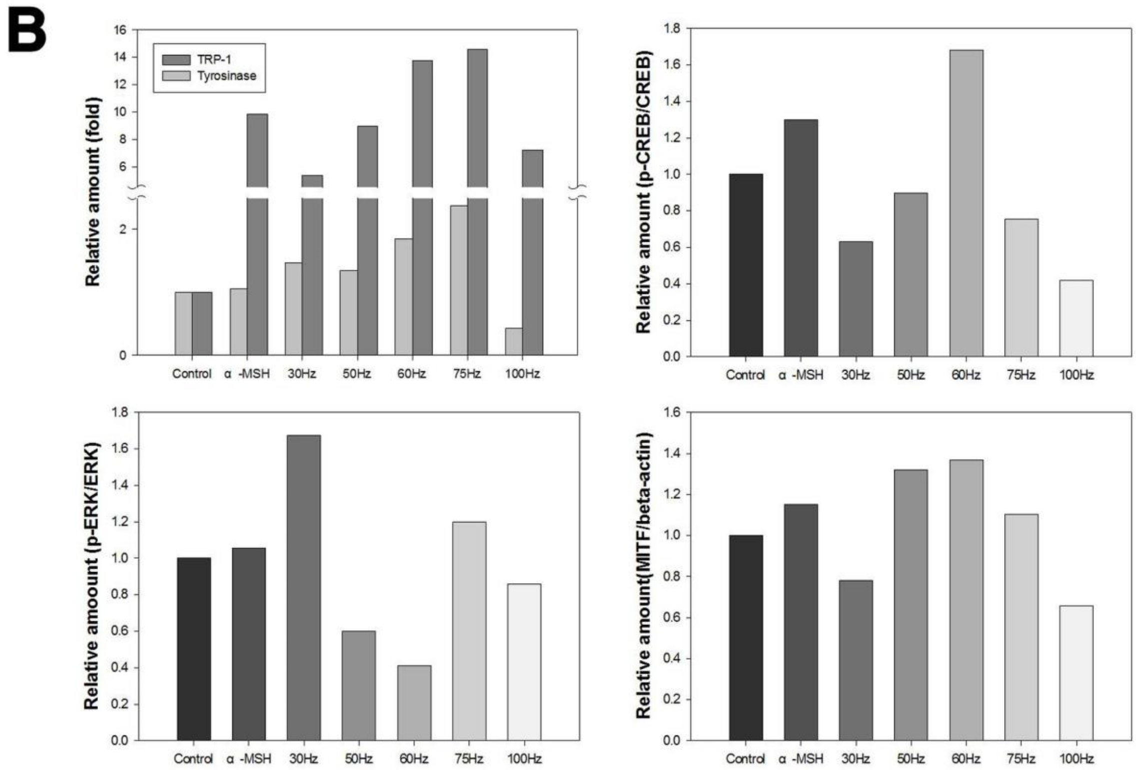

B
Relative amount (fold)
TRP-1
Tyrosinase
Control
α -MSH
30Hz
50Hz
60Hz
75Hz
100Hz
Relative amount (p-CREB/CREB)
Relative amount (p-ERK/ERK)
Relative amount(MITF/beta-actin)

Fig. 8B

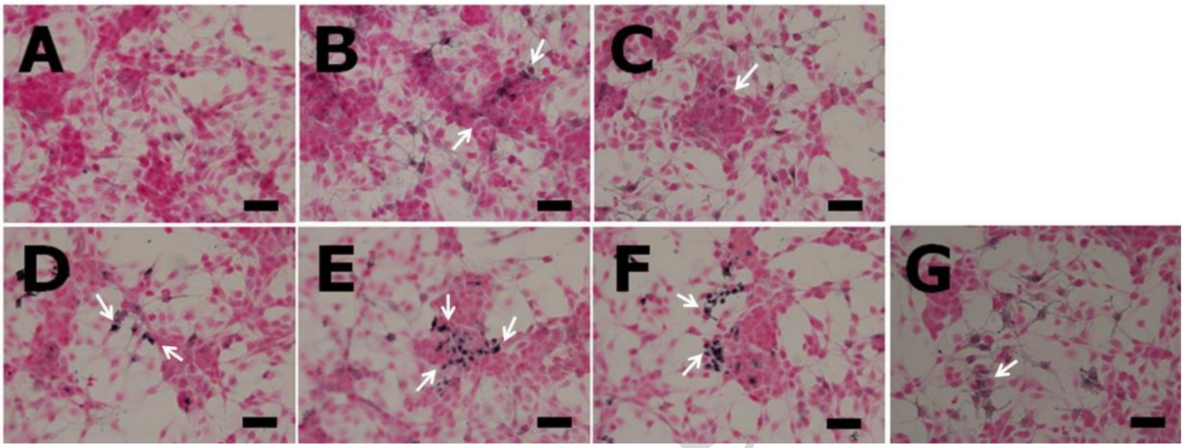
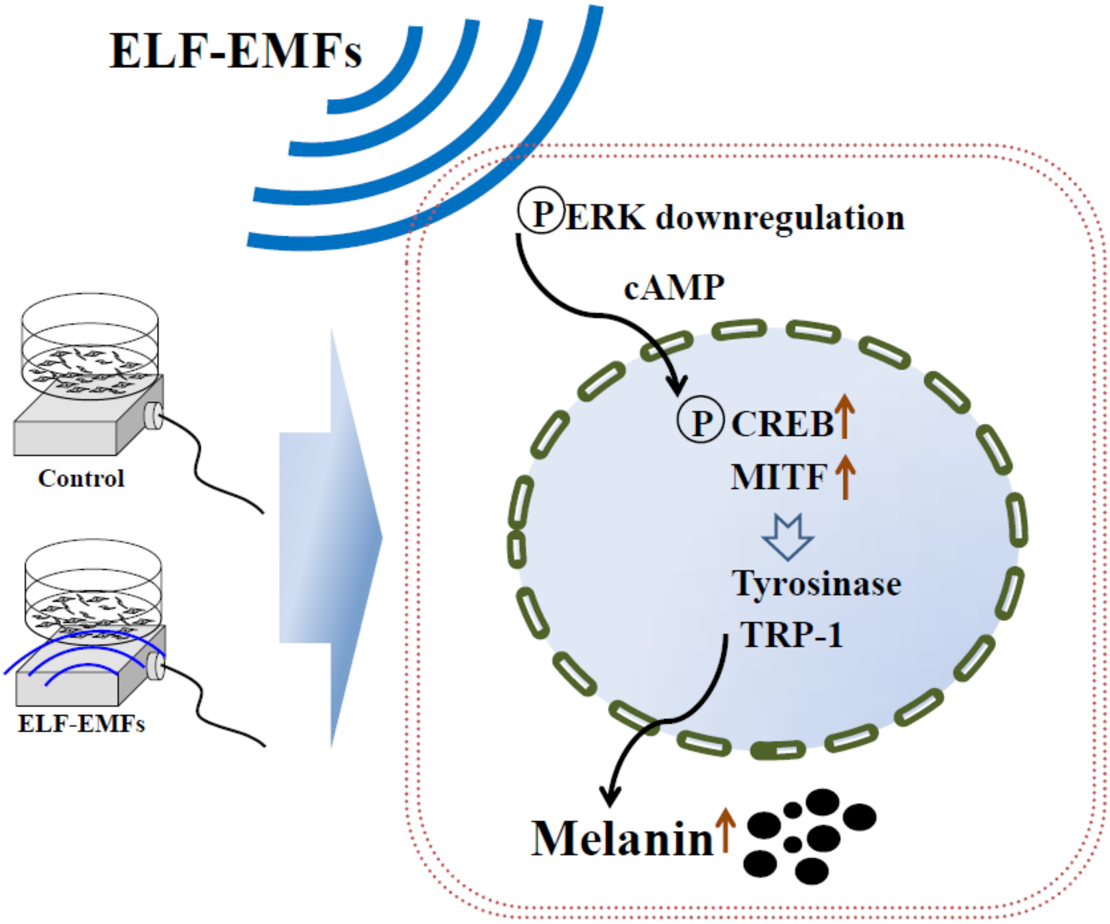


Fig. 9

Table 1

staining	Control	MSH	30Hz	50Hz	60Hz	75Hz	100Hz
Fontana Masson	−	++	+	++	+++	+++	+



Graphical abstract